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Studies on the Conversion of Proinsulin to Insulin

I. CONVERSION *IN VITRO* WITH TRYPSIN AND CARBOXYPEPTIDASE B*

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SUMMARY

Proinsulin can be rapidly and quantitatively cleaved by a combination of pancreatic trypsin and carboxypeptidase B *in vitro* to yield intact insulin, the C-peptide, and free arginine and lysine. No significant desalanylation or further degradation of the insulin occurs under the specified conditions. This *in vitro* system appears to provide a useful model for studies of the *in vivo* converting enzyme system localized in the β-cells of the islets of Langerhans.

The mechanism for the proteolytic conversion of proinsulin to insulin is of special interest since, unlike most zymogen activations, this conversion takes place within the β-cells prior to the secretion of the insulin (1, 2). Several lines of evidence summarized elsewhere suggest that the conversion process is localized to the Golgi apparatus or the newly formed secretory granules or "progranules" in the β-cell (3–5). We recently have presented evidence that a partially purified islet granule fraction can carry out the conversion of endogenously labeled rat proinsulin with release of insulin and the bulk of the proinsulin connecting polypeptide segment (6, 7). These findings are in agreement with observations of Clark *et al.* (8) and Rubenstein *et al.* (9) that the connecting peptide (C-peptide) and insulin are present in equal amounts in the pancreas and are secreted together into the incubation medium *in vitro* or the circulation *in vivo*.

The structure of several mammalian proinsulins have now been elucidated and the polypeptide chain in all of these appears to be ordered similarly as follows: NH_2-B chain–Arg–Arg–C-peptide–Lys–Arg–A chain-COOH (10–13). Studies of the intermediate fraction of bovine proinsulin, which coexists with the intact form of proinsulin in crystalline preparations of bovine insulin (12), indicate the existence of a cleavage mechanism in which the pairs of basic residues linking the C-peptide to the A and B chains are excised with liberation of native insulin and the free C-peptide without the terminal basic residues (Fig. 1). Extraction and purification of C-peptides from human (13), monkey (13), bovine (14), ovine,[1] porcine (15), and canine[1] pancreas all confirm that the C-peptide is a major transformation product that occurs in the pancreas on an equimolar basis with insulin. The human C-peptide has been utilized as a readily available material for the amino acid sequence analysis of human proinsulin, since sufficient amounts of human proinsulin for sequence analysis have not yet been obtained (13).

In previous reports we postulated that the conversion of proinsulin to insulin via the known intermediate forms, and accompanied by the release of C-peptide lacking basic residues, could be accomplished by the combined action of enzymes having properties similar to pancreatic trypsin and carboxypeptidase B (12, 14, 16, 17). This supposition was based on our earlier observation (10, 16), later confirmed by Chance, Ellis, and Bromer (11) and Grant and Reid (18), that proinsulin can be rapidly converted to an insulin-like component (desalanyl insulin) by trypsin, although native mammalian insulin cannot be produced by this protease. It was observed that the Arg–Gly and Arg–Glu bonds in the carboxyl- and amino-terminal regions, respectively, of the bovine or porcine proinsulin-connecting polypeptide segment are split very rapidly by trypsin, whereas further cleavage of insulin between Lys–Ala (B_{29}-B_{30}), and at other sites, proceeds much more slowly (12, 19). The more rapid cleavage of the B chain Lys–Ala bond than the Arg–Gly (B_{22}-B_{23}) bond in proinsulin is opposite to the relative rates of cleavage of these bonds in insulin (20). Wang and Carpenter suggest that this altered behavior may be due to enhancement of the rate of cleavage at the Lys–Ala position by the adjacent pair of arginine residues that are present only in proinsulin or some of its tryptic fragments.

In this report we demonstrate that the addition of an excess of pancreatic carboxypeptidase B to the trypsin during the conversion of proinsulin *in vitro* results in the rapid and quantitative transformation of the proinsulin to native insulin and C-peptide with the liberation of 3 residues of free arginine and 1 residue of free lysine. Further cleavage of the insulin at the Lys–Ala position in the B chain or at other sites was not observed under the conditions employed.

EXPERIMENTAL PROCEDURE

Bovine proinsulin (zinc-free) was isolated in high purity as described previously (16). Purified porcine trypsin, kindly sup-

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[1] J. D. Peterson, S. Nehrlich, and D. F. Steiner, unpublished data.

It was essential to ascertain the effects of carboxypeptidase B on insulin under these conditions of pH and enzyme concentration. Digests were prepared containing 0.75 μmole per ml of zinc-free bovine insulin with the same buffer and conditions as above. With either 2 or 20 μg per ml of carboxypeptidase B release of asparagine or alanine from insulin could not be detected during incubations extending up to 40 min. With 90 μg per ml of carboxypeptidase B approximately ½ residue of alanine and a trace of asparagine were released after incubation for 2 hours at 37°.

Several experiments were then carried out with various ratios of trypsin to carboxypeptidase B, using intact bovine proinsulin as the substrate. From earlier experiments we knew that with trypsin (100 μg per ml) and proinsulin (~1 μmole per ml) at pH 8.2, cleavage of the Arg–Gly (positions 59 and 60) and Arg–Glu (positions 32 and 33) bonds occurred very rapidly, reaching completion within the first 2 min of incubation (14). Accordingly the initial concentration of trypsin was lowered considerably (8.0 μg per ml); the proinsulin concentration was maintained at 1.2 μmoles per ml. Carboxypeptidase B was added at a final concentration of 1.4 μg per ml, and the mixture was incubated for 110 min at 37°. During this time amino acid analysis of aliquots from the digest revealed only very slow release of free arginine and lysine in a ratio of 1:1 indicating that very slow cleavage at positions 59 and 60 was occurring, perhaps due to the lower pH used here, pH 7.5. At 110 min the trypsin concentration was increased 5-fold which resulted in the rapid appearance (2 to 3 min) of diarginyl insulin, which tends to form an isoelectric precipitate at pH 7.5. The carboxypeptidase B concentration therefore was doubled quickly and the turbidity gradually cleared during the next 2 min, after which time the reaction was stopped by acidification. Analysis of this digest revealed an arginine to lysine ratio of 2.39 instead of the expected ratio of 3:1. Thin layer chromatography revealed the presence of a small amount of material having the mobility of Ala–Arg. Thus the increase in trypsin concentration during incubation was excessive and evidently caused dealanylation of the insulin-Arg–Arg before carboxypeptidase B could protect the product by removing the terminal arginine residues.

Using these results as a guide an intermediate concentration of trypsin was chosen to lessen the tendency for dealanylation, and a higher amount of carboxypeptdase B was added to ensure the rapid removal of terminal basic residues as these appeared. Proinsulin (0.8 μmole per ml) was incubated with trypsin (25 μg per ml) and carboxypeptidase B (12.5 μg per ml) at 37° and pH 7.5. Aliquots were removed for thin layer chromatography, amino acid analysis, and polyacrylamide gel electrophoresis at 10, 20, 40, and 80 min. Under these conditions the reaction was essentially complete after 10 min of incubation as indicated by the stoichiometric release of arginine and lysine in a ratio of 3:1 and the presence of only a single band at the position of insulin on gel electrophoresis. Analysis of the acidic and neutral residues after 40 min of digestion revealed no free asparagine or alanine. No Ala–Arg was detected by thin layer chromatography at any time.

This experiment was repeated using 690 μg of proinsulin and the same concentrations of carboxypeptidase B and trypsin as

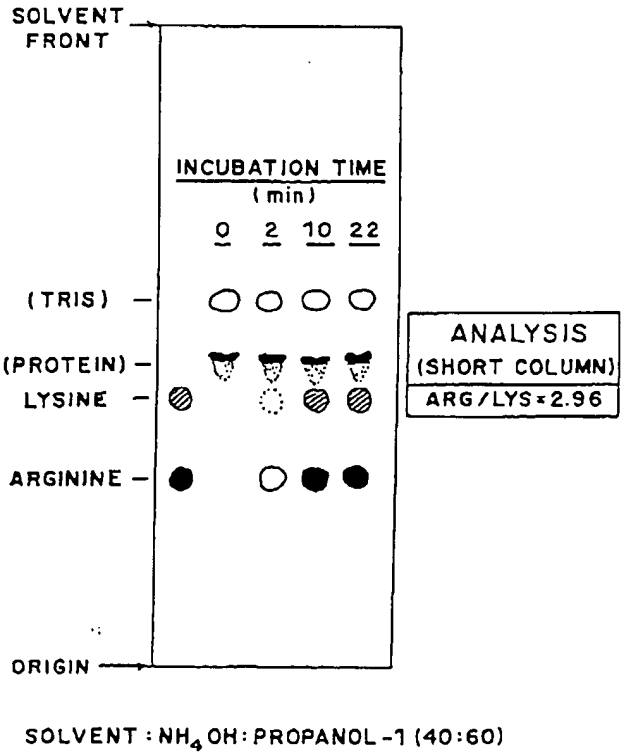

FIG. 2. Thin layer cellulose chromatogram of proinsulin conversion products illustrating the release of free arginine and lysine. (Alanylarginine in this system is found just below lysine; none was detectable in this experiment.)

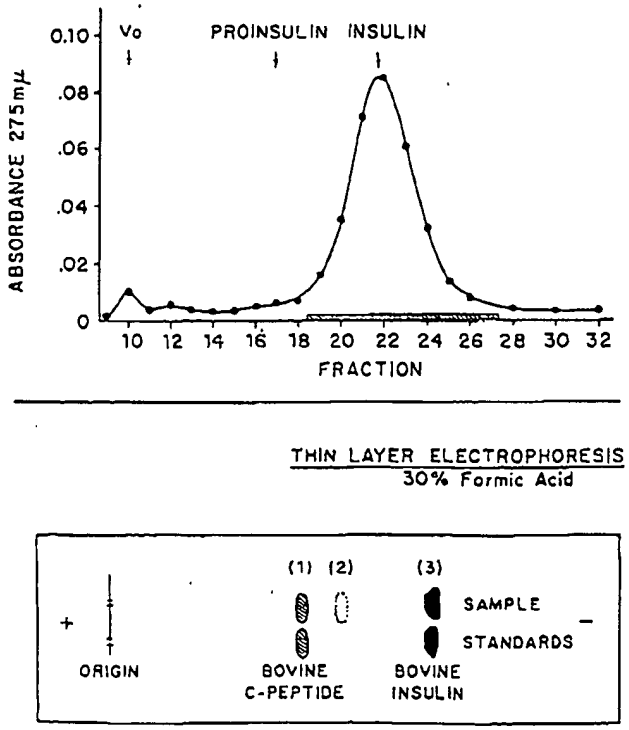

FIG. 3. *Upper panel*, Bio-Gel P-30 gel filtration elution profile of converted proinsulin (fraction volume = 1.4 ml). *Lower panel*, thin layer cellulose electrophoretogram of the material from Fractions 19 to 27 of the gel chromatogram. (See "Experimental Procedure" for further details.)

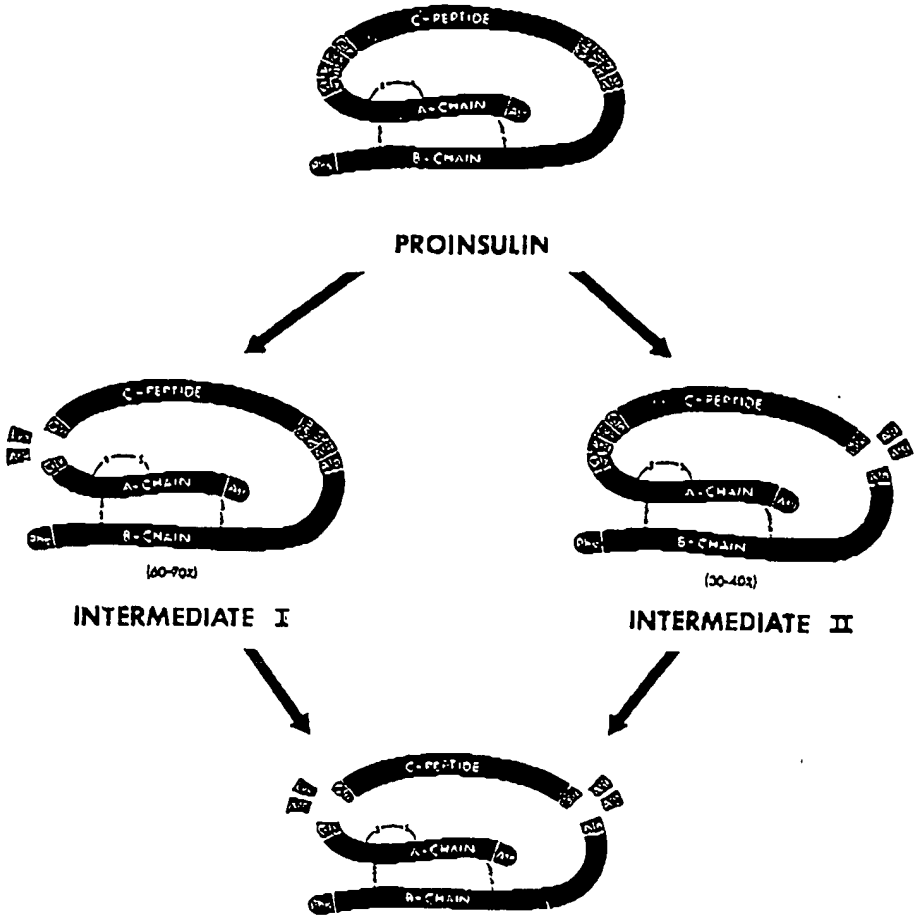

FIG. 1. Simplified representation of the conversion of bovine proinsulin to insulin and C-peptide *in vivo* via the known major intermediate forms of bovine proinsulin (12). (See Fig. 5 for a more detailed scheme.)

plied by the Novo Company (Copenhagen), was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone by the procedure of Wang and Carpenter (21). Carboxypeptidase B (diisopropyl fluorophosphate-treated) was purchased from Worthington.

Gel filtration was carried out on columns (1 × 50 cm) of Bio-Gel P-30 (BioRad Laboratories, Inc.) equilibrated with 3 M acetic acid. Polyacrylamide gel electrophoresis was carried out by the method of Ornstein and Davies (22). Amino acid analyses were performed on a Beckman 120C amino acid analyzer essentially as described by Spackman (23). Samples for analysis were hydrolyzed for 24 hours with 6 N HCl containing 0.25% phenol at 110° in sealed evacuated glass tubes. In some instances aliquots from proteolytic digests were analyzed without prior hydrolysis to detect free asparagine, alanine, arginine, and lysine.

Thin layer chromatography was carried out on sheets of Eastman 6064 cellulose using concentrated NH_4OH-propanol-1 (40:60) as the solvent, a modification of system G of Stewart and Young (24). Electrophoresis was carried out on sheets of Eastman thin layer cellulose in 30% formic acid for 5 hours at room temperature in a closed box with a potential gradient of 7.5 volts per cm (25). Peptides were eluted from thin layer plates by scraping free the dry cellulose powder and extracting it several times with 50% acetic acid in tapered centrifuge tubes. Insulin was crystallized with zinc at pH 6.2 (26).

RESULTS

In order to determine the approximate rate of cleavage by carboxypeptidase B of carboxyl-terminal basic residues from peptides similar to those occurring in proinsulin and its intermediate forms, preliminary trials were carried out using a tryptic peptide of proinsulin consisting of C-peptide–Lys–Arg (12, 14) as a substrate for carboxypeptidase B. Release of basic residues was followed both by thin layer chromatography and by direct amino acid analysis of portions of the digests. These incubations were carried out at 37° in 0.1 M Tris-HCl buffer, pH 7.6. The substrate concentration was 0.5 μmole per ml. After incubation for 20 min, release of arginine and lysine was complete with a carboxypeptidase B concentration of 2 μg per ml. With 20 μg per ml of carboxypeptidase B, the reaction was complete within the first few minutes of incubation. Further degradation of the C-peptide did not occur under these conditions even with 200 μg per ml of carboxypeptidase B.

TABLE I
Amino acid composition of proinsulin conversion products

Amino acid	Fraction 1 C-peptide	Fraction 2 Monobasic C-peptide[a]	Fraction 3 Insulin
	residues/molecule[b]		
Lysine	Trace	0.18	0.85 (1)
Histidine		Trace	1.87 (2)
Arginine		0.76	1.00 (1)
Aspartic acid			3.07 (3)
Threonine			0.94 (1)
Serine			2.81 (3)
Glutamic acid	6.21 (6)	6.34 (6)	7.58 (7)
Proline	4.16 (4)	4.14 (4)	1.17 (1)
Glycine	7.75 (8)	8.08 (8)	3.84 (4)
Alanine	2.06 (3)	2.86 (3)	2.96 (3)
Half-cystine			3.95[c] (6)
Valine	1.77 (2)	1.57 (2)	4.56 (5)
Isoleucine			0.50 (1)
Leucine	3.04 (3)	2.99 (3)	6.04 (6)
Tyrosine			3.87 (4)
Phenylalanine			2.77 (3)
Total fraction (nmoles)	17.85	3.68	22.04

[a] Contains both C-peptide–Lys and Arg–C-peptide.
[b] 24 hour hydrolysis; 6 N HCl, 0.25% phenol; 110°. Theoretical value is given in parentheses.
[c] No precautions were taken to ensure full recovery of half-cystine; this value is typical for similar hydrolysates of insulin.

in the previous experiment. Aliquots from the digest spotted at 2, 10, and 22 min of incubation at 37° again showed the reaction to be essentially complete after 10 min but not after 2 min (Fig. 2). The digest was taken up in 8 M acetic acid and gel filtered on a column (1 × 50 cm) of Bio-Gel P-30. A single peak of ultraviolet-absorbing material was obtained which had the same elution volume as insulin (Fig. 3, *upper panel*). The fractions containing this component and the C-peptide were collected as shown in Fig. 3 and were evaporated to dryness. The residue was dissolved in a small volume of 50% acetic acid, applied in a 5-cm band to a thin layer cellulose plate moistened with 30% formic acid, and subjected to electrophoresis in this solvent. Marker strips were removed from the dried plate and stained with ninhydrin. Three ninhydrin-positive components which migrated toward the cathode were found (one in relatively much smaller amounts) as shown in the diagram in the *lower panel* of Fig. 3. These were eluted from the cellulose and their compositions determined. The compositional data shown in Table I confirm that the major products were insulin and the C-peptide. Moreover, the insulin contained the expected 3 residues of alanine, and the C-peptide was free of basic residues. The minor component on the electrophoretogram consisted mainly of C-peptide having amino-terminal arginine, confirming our previous finding (14) that trypsin tends to cleave between Arg–Arg (positions 31 and 32) at a rate about 10 times lower than the rate of cleavage on the carboxyl side of arginine (position 32).

The remaining insulin fraction after electrophoresis was crystallized with zinc at pH 6.2 using a micro method (26). Typical rhombohedral crystals were obtained which could not be distinguished from the crystals produced by ordinary bovine insulin. A polyacrylamide gel electrophoretogram of the insulin derived from proinsulin is shown in Fig. 4.

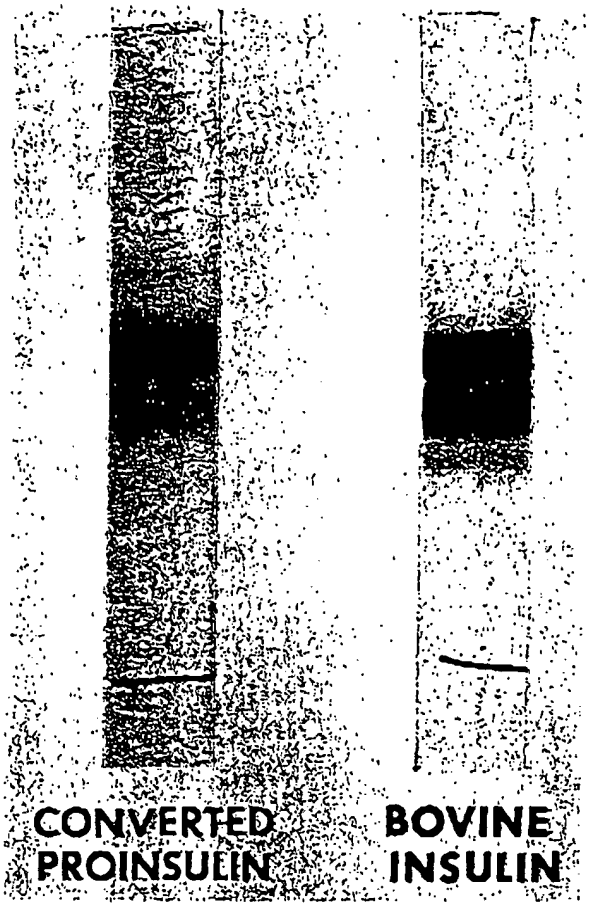

FIG. 4. Polyacrylamide gel electrophoretograms (pH 8.4) of the proinsulin conversion product and of bovine insulin. The insulin preparation used here contains several faster migrating deamido components; these are present at a much lower level in the insulin derived from proinsulin. The tracker dye position is indicated by the *wire strands below*.

DISCUSSION

The data presented here show that, as hypothesized earlier, trypsin and carboxypeptidase B can quantitatively convert proinsulin to insulin accompanied by the liberation of C-peptide and stoichiometric quantities of free lysine and arginine. Close attention to the conditions of incubation appears to be quite important for the success of this method; particularly critical is the ratio of carboxypeptidase B to trypsin. This is apparently because trypsin tends to cleave the Lys–Ala bonds in the insulin B chain more rapidly when the 2 arginine residues at positions 31 and 32 are still present than after these have been removed (20). To circumvent this difficulty *in vitro* the trypsin concentration must be made rate-limiting relative to the carboxypeptidase B so that intermediates having terminal basic residues do not accumulate.

A hypothetical reaction sequence for the trypsin- and carboxypeptidase B-mediated conversion of proinsulin is shown in Fig. 5. This scheme not only leads to the known products of

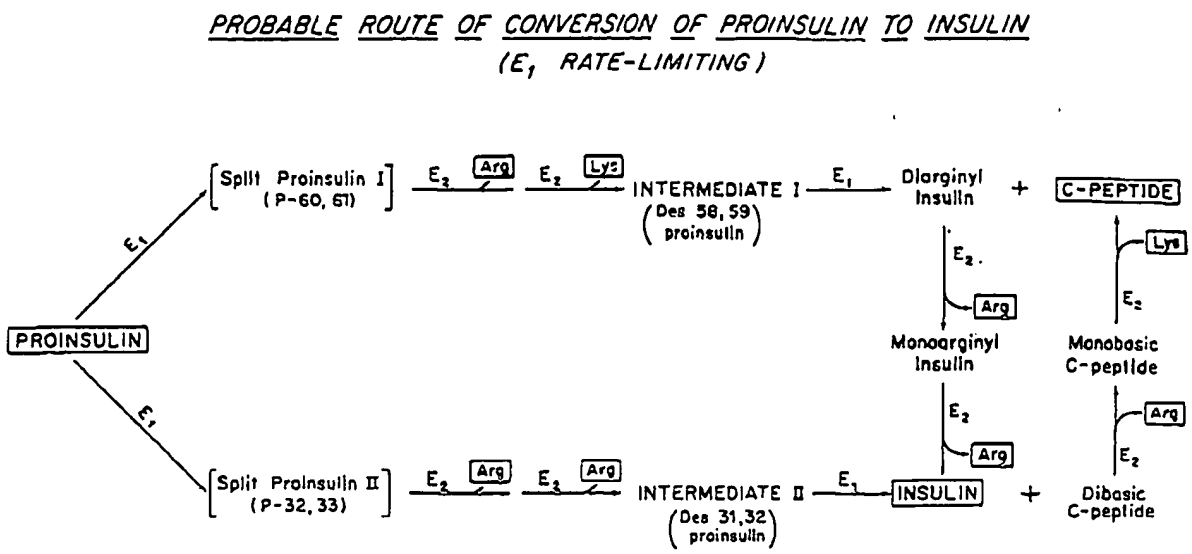

FIG. 5. Schematic representation of the hypothetical *in vivo* conversion pathway of proinsulin to insulin by enzymes having trypsin-like and carboxypeptidase B-like specificities based on the *in vitro* studies reported here. The accumulation of intermediates I and II would be expected in this system with the trypsin-like activity limiting. The major products would be native insulin and the C-peptide. The upper pathway would predominate since cleavage at positions 60 and 61 is more rapid than cleavage at positions 32 and 33 (19).

proinsulin transformation that have been isolated and identified (14), but it also favors the accumulation of certain intermediate forms of proinsulin rather than others, and these correspond to the intermediates which have been found in greatest abundance in the crude proinsulin fraction separated from crystalline bovine insulin (12). These facts, taken together with the known variability of the carboxyl-terminal residue of the B chain in insulin (25), the presence of small amounts of mono-arginyl insulin in commercial insulin preparations,[3] and additional biosynthetic evidence for the stepwise removal of the basic residues during the conversion of proinsulin in intact islets of Langerhans (27), all argue strongly against the existence of novel converting enzymes, particularly highly specific endodipeptidases. In view of their close developmental relationship (28) and the probable evolutionary derivation of the endocrine and exocrine pancreas from the intestinal epithelium, as well as the pre-eminent role played by trypsin in the activation of all of the exocrine pancreatic zymogens, it seems reasonable to assume that the proinsulin-converting enzymes are closely related to some of the exocrine enzymes (17). In the case of many fish insulins, trypsin or a similar enzyme acting alone could apparently accomplish the conversion of the proinsulin since these insulins are similar in structure to mammalian dealanyl insulins (25, 29).

Preliminary studies of the conversion of rat proinsulin labeled with [^{3}H]arginine by disrupted secretory granules prepared from islets of Langerhans indicate the presence of a carboxypeptidase B-like activity in addition to a low level of trypsin-like activity.[4]

[3] W. Kemmler and D. F. Steiner, unpublished data.
[4] W. Kemmler, J. Borg, and D. F. Steiner, unpublished data.

Further studies of this cell-free system from islets of Langerhans using the trypsin- and carboxypeptidase B-mediated conversion *in vitro* as a model should assist in the elucidation of the conversion mechanism. These findings also provide a highly efficient means for the conversion of proinsulin to insulin, which could be of considerable practical value should the laboratory synthesis of insulin via proinsulin be undertaken.

Addendum—In the interval since this paper was submitted for publication a report has appeared (30) describing the isolation from bovine pancreas of an anionic trypsin-like enzyme that converts proinsulin to insulin. This enzyme preparation apparently cleaves on the carboxyl side of the alanine residue at position 30 of the B chain sequence and at the arginine residue linking the connecting polypeptide to the amino-terminal glycine residue of the insulin A chain to liberate insulin. Other peptide products were not characterized. Although some free arginine was liberated in this reaction, free lysine was not found, suggesting that one of the cleavage products is the C-peptide bearing either lysine or lysylarginine at the carboxyl terminus. Since the C-peptide, which has no basic residues, is the product normally found with insulin in the pancreas (14), it seems likely that additional, or possibly different, enzymes participate in the cleavage of proinsulin *in vivo*.

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